

Short communication

Determination of morphine and its analogues in urine by on-line coupled reversed-phase liquid chromatography–gas chromatography with on-line derivatization

Tuulia Hyötyläinen, Helena Keski-Hynnälä, Marja-Liisa Riekkola*

Laboratory of Analytical Chemistry, Department of Chemistry, University of Helsinki, P.O. Box 55, FIN-00014 University of Helsinki, Finland

Received 16 October 1996; revised 30 January 1997; accepted 10 February 1997

Abstract

An on-line coupled reversed-phase liquid chromatography–gas chromatography (RPLC–GC) method requiring minimal manual sample preparation was developed for the determination of morphine, codeine, heroin, dihydrocodeine and ethylmorphine in urine. A loop-type interface and concurrent eluent evaporation technique were used in the coupling, and the aqueous phase was changed to organic solvent by continuous liquid–liquid extraction before transfer to the GC. The two phases were separated with a sandwich-type phase-separator. The analytes were also derivatized on-line before GC separation. The LC was used for clean-up, and the GC for the final separation and detection of the analytes. Total analysis time was less than 60 min, which is much shorter than with traditional analysis methods. A marked increase in the recoveries in liquid–liquid extraction (LLE) was obtained by heating the aqueous eluent and the extraction coil. Linearity and repeatability of the method were good and the limits of quantification for the analytes were between 61 and 92 ng/ml.

Keywords: Derivatization, LC–GC; Urine; Morphine; Codeine; Heroin; Dihydrocodeine; Ethylmorphine

1. Introduction

The extensive use of morphine and its analogues as analgesics together with the widespread abuse of these drugs has necessitated the development of rapid and sensitive methods for the detection of opiates in biological samples. Assays of opiates are of importance both in clinical medicine and in forensic toxicology. In addition, in many countries job applicants are tested for drugs. Various techniques have been developed for these purposes, including immunoassay [1,2] and electrophoretic [3–5] and chromatographic techniques [6–14]. Of these,

immunoassay systems offer the sensitivity required, but they rarely differentiate individual opiates from each other. Electrophoresis is a relatively new technique in this area and not yet widely used in routine analysis. Chromatographic techniques are therefore the most widely used for analysing opiates in biological matrices. Thin layer chromatography (TLC) [6] usually is used only for screening purposes while liquid chromatography (LC) [7,8] and gas chromatography (GC) [9–13] are also applied for quantitative analysis. Supercritical chromatography (SFC) [14] has been used in lesser extent in the analysis of opiates.

A major drawback of these otherwise superior chromatographic techniques is the time-consuming

*Corresponding author.

sample preparation. The most common sample preparation methods for biological samples are liquid–liquid extraction (LLE) and solid-phase extraction (SPE). Through careful choice of solvent, LLE allows the extraction of analytes from most endogenous components, but the method is tedious and sufficient recovery of analytes often requires successive extractions and large volumes of solvents. In comparison with LLE, SPE is simpler and faster and much less solvent is required. In addition to these clean-up procedures, GC methods frequently require derivatization of polar and adsorptive groups of the analytes before the separation. Most of these sample preparation steps are carried out manually, which is not only laborious but often also imprecise, and the risks of sample loss and contamination are increased. For all these reasons, much attention has lately been given to the development of fast and simple automated sample handling procedures, such as automated preparation modules in GC [15], on-line SPE–GC [16], LLE–GC [17], column-switching techniques in LC [18], and hyphenated chromatographic techniques such as LC–LC [19] and LC–GC [20–23].

Coupling together of two different chromatographic instruments allows exploitation of the best features of both. In LC–GC coupling, LC is used for clean-up and/or fractionation of the sample while the actual separation is done by more efficient GC. Thus the large sample capacity and high resolving power of LC can be combined with high separation efficiency and sensitive detection of GC. An additional benefit of LC–GC coupling is that the whole sample fraction can be injected into the GC unlike in traditional techniques. Although it is much easier to couple normal-phase than reversed-phase LC to GC, RPLC–GC coupling offers a wider application range. Moreover, because there are problems in injecting large volumes of aqueous eluents directly into the GC column, the RPLC–GC combination demands special approaches. Usually the RP-eluent is changed to a suitable organic solvent before GC analysis by using a trapping column, SPE column, or on-line liquid–liquid extraction [24]. Choice among the several types of interfaces available for the RPLC–GC coupling depends on the analytes. The ideal interface for drugs, which tend to be relatively polar and non-volatile, is the loop-type interface using fully concurrent eluent evaporation (FCSE).

In this work, an on-line coupled RPLC–GC system was used for determination of morphine and its analogues in urine. The aim was to develop a fast method with minimal manual sample preparation. The coupling was based on on-line LLE, a sandwich-type phase separator, and a loop-type interface, and the analytes were silylated with bis-(trimethylsilyl)acetamide (BSTFA) on-line before the GC separation. Concurrent eluent evaporation mode was used during the transfer step and the excess of solvent vapors was removed via a solvent vapor exit. The sample clean-up was done in the LC part of the system and the only manual sample preparation step was filtration of the sample. Linearity, sensitivity and repeatability of the method were studied.

2. Experimental

2.1. Materials

All solvents were HPLC grade. Acetonitrile, methanol, dichloromethane, boric acid, ethyl acetate and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Codeine hydrochloride, ethylmorphine hydrochloride, heroin hydrochloride, hydrocodeine bitartrate and morphine hydrochloride were donated by the Crime laboratory of the Finnish National Bureau of Investigation (Vantaa, Finland). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and BSTFA+1% trimethylchlorosilane (TMCS) were from Merck.

2.2. Instrumentation and conditions

The coupled LC–GC system has been described earlier [20,21]. The liquid chromatograph was a Hewlett-Packard 1090 with diode array detector. The 6-port valve for backflushing the LC column was from Rheodyne. Organic solvent was delivered by a Jasco pump. The extraction coil material was PEEK (3 m×0.75 mm I.D.). The sandwich-type phase separator was made in our laboratory [25]. The LC–GC interface consisted of a 10-port Rheodyne valve equipped with a 940- μ l sample loop and an additional 78- μ l loop for the derivatization reagent. The GC was a Carlo Erba Mega Series 5300 equipped with a flame ionization detector.

LC separations were performed on a 20 mm×2.1

mm I.D. column dry-packed with Capcell Pak C₁₈ SG-120, 5 µm (Shiseido, Japan). The LC mobile phases were 0.05 M boric acid adjusted to pH 10.0 with NaOH, and boric acid–acetonitrile (76:24, v/v). Mobile phases were filtered and degassed before use. Flow-rate of the mobile phase was 0.8 ml/min. Diode array detection was at 240, 254 and 280 nm.

Columns used in GC separation were a 3 m×0.32 mm I.D. fused-silica DPTDMS deactivated retention gap (BGB Analytic, Switzerland), a 3 m×0.32 mm I.D. fused-silica retaining precolumn coated with 0.25 µm film of BGB-5 (BGB Analytik) and an 18 m×0.32 mm I.D. fused-silica capillary column coated with 0.1 µm film of BGB-5 (BGB Analytik), all connected via press fit connectors. The detector temperature was 300°C. The carrier gas was helium and the flow-rate 7.3 ml/min.

2.3. Analysis procedure

LC clean up: 1–3 min: washing with boric acid. 3–6 min: Elution with boric acid–ACN (76:24, v/v). On-line LLE: extraction solvent: CH₂Cl₂. Separation of the organic and aqueous phases: sandwich-type separator, 0°C. Collection of the sample fraction, transfer of the sample fraction to GC: solvent vapor exit open, oven temperature 90°C, evaporation of the solvent (15 min), derivatisation in the retention gap (2 min), solvent vapor exit closed. GC separation: 90°C→160°C→225°C→300°C at 20, 4 and 25°C/min, respectively.

2.4. Samples

Standard solutions of the drugs were made by dissolving an appropriate amount of each in methanol to give a concentration of 1 mg/ml. The solutions were stored at 4°C. Spiked samples were prepared by adding standard solutions to drug-free urine, which had been collected from healthy volunteers after a caffeine-free diet lasting 2 weeks. Urine samples were diluted 1:1 (v/v) with 0.05 M boric acid (pH 10.0) and filtered. A 100-µl volume was injected into LC loop of corresponding volume.

2.4.1. Off-line derivatization procedure

The solution of analytes (concentration of each 1 µg/ml) in boric acid buffer (2 ml) was extracted twice with two portions of 1-ml volume of dichloro-

methane and the solution was evaporated to dryness under a flow of nitrogen. A 135-µl volume of BSTFA was added to the residue and the capped test tube was heated at 70°C for 30 min. Finally the mixture was diluted with 2 ml of dichloromethane.

3. Results and discussion

The goal of the LC separation was to similar to solid-phase extraction: to separate the analytes not from each other but from the endogeneous matrix compounds. A short column of 2 cm×2.1 mm I.D. proved suitable for this purpose. The LC procedure consisted of a washing step followed by an elution step. A 3-min washing with the buffer was sufficient to remove most of the disturbing compounds. In the elution step, the concentration of the organic solvent, acetonitrile, was optimized in the range of 20–30% (v/v) to obtain as narrow a zone as possible. No sample loss was noticed in the washing step.

In the on-line liquid–liquid extraction step the aqueous eluent was changed to suitable organic solvent for the GC separation. This also served as an additional clean-up step. Boric acid at pH 10 was chosen as the aqueous eluent and dichloromethane as the extraction solvent. In our earlier studies we found that equal flow-rates of 0.8 ml/min for the organic and aqueous phases gave the best extraction efficiency and separation in the phase-separator [20,21].

The effect of temperature on the extraction was studied by heating the aqueous eluent and the extraction coil. It was necessary to heat the eluent, not just the coil system, because the heat transfer through the PEEK material is not very efficient. Since the LC system is closed and the pressure inside the system exceeds the ambient pressure, the temperature could be increased above the boiling point of the extraction solvent. The boiling point of dichloromethane is 39°C at ambient pressure but the system could be heated to 55°C. At this temperature, however, the sample zones became much broader due to enhanced diffusion and, furthermore, the efficiency of the phase-separator was decreased. A lower temperature of 45°C was accordingly chosen for the analysis. The efficiencies of on-line extraction for the analytes at 45°C were as much as six times as good as the efficiencies at ambient temperature.

Compared with traditional, manual liquid–liquid extraction, the efficiency of on-line extraction was better for all the opiates except ethylmorphine (Table 1).

To enhance the evaporation rate of the solvent, a higher flow-rate was used for the transfer of the sample fraction and derivatization reagent, than during the actual separation. Carrier gas inlet pressures were tested in the range 1.5–2.1 bar to give flow-rates of 7.2–12.3 ml/min. The inlet pressure was set 0.2 bar lower during the separation step. Although the evaporation time was reduced to 8 min at the higher inlet pressure (2.1 bar), the separation was negatively affected and therefore an inlet pressure of 1.5 bar (flow-rate 7.3 ml/min) was chosen. The oven temperature was optimized for the transfer step by taking into account the conditions required both for concurrent eluent evaporation and for the on-line derivatization reaction. The effect of temperature was studied in the range 88–94°C and 90°C was selected as best for both separation and the derivatization reaction.

Although it is also possible to analyse opiates by GC without derivatization, most such analysis procedures are limited by the phenolic properties of the opiates, which cause non-linear adsorption to the chromatographic column, with resultant tailing of the peak and irreversible adsorption. Various methods have been developed to derivatize the free hydroxyl groups of these compounds [9–13], of these silylation has good characteristic for an on-line derivatization and it was accordingly chosen as the derivatization method. Two silylation reagents were tested: BSTFA with 1% TMCS, and BSTFA. BSTFA/TMCS had a negative effect on the surface of the retention gap, whereas no such effect was

noticed with pure BSTFA. The latter was chosen for further studies. As mentioned above, the temperature during the transfer of the sample fraction was also optimized with a view to efficient and fast derivatization. It can be assumed that the derivatization takes place in the retention gap after the solvent has evaporated. A short period of two min after the evaporation of the solvent was sufficient for the derivatization reaction. For the optimization of the amount of reagent, BSTFA was diluted with dichloromethane in the concentration range of 10–50%. The effect of varying BSTFA concentration was studied with standard samples containing 1 µg/ml of each opiate. The optimal concentration was found to be 10% BSTFA in CH₂Cl₂ for 1 µg/ml of analyte. This was in slight excess, using 8% instead of 10% of the reagent gave same derivatization yield, but the repeatability was not as good with lower concentration. Optimization of the concentration was necessary not only for repeatable results but because large excess of silylation reagent disturbed the separation of the analytes and contaminated the detector with silica deposits. The on-line derivatization was compared with off-line derivatization with the same reagent (Table 1). The reaction yields were comparable.

As can be seen from Fig. 1, the clean-up procedure was effective and there were no interfering compounds from the urine matrix in the chromatogram. The extra peaks are due to the excess of derivatization reagent. To evaluate the applicability of the analysis procedure to quantitative analysis, we studied repeatability, linearity and limits of detection. The calibration curves showed linear relationships between peak areas and the analyte concentrations in the range 0.09–15 µg/ml (Table 2). The minimum

Table 1
Comparison of efficiency of manually made and on-line liquid–liquid extractions and off-line and on-line derivatization efficiencies

	Manual LLE	On-line LLE at 20°C	On-line LLE at 45°C	Off-line derivatization	On-line derivatization
Dihydrocodeine	1	0.6	3.8	1	0.7
Codeine	1	0.4	1.4	1	1.1
Ethylmorphine	1	0.7	0.7	1	0.8
Morphine	1	0.4	– ^a	1	– ^a
Heroin	1	0.6	1.0	1	1.9

^a Not measured.

Calculated with respect to off-line extraction and derivatization. Calculated from six replicant runs.

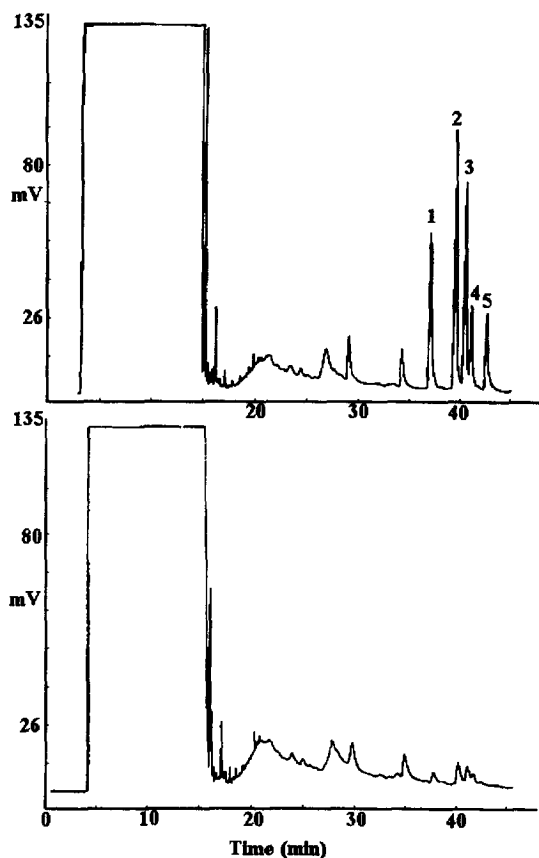


Fig. 1. GC chromatograms of (top) urine spiked with the opiates and (bottom) blank urine. Peaks: (1) dihydrocodeine, (2) codeine, (3) ethylmorphine, (4) morphine and (5) heroin. Concentration of the analytes was 3 $\mu\text{g/ml}$.

concentrations of the analytes in urine that could be determined were 61–92 ng/ml. The flame ionizor detector that we used is not very sensitive for the opiates. With a more selective detector, e.g., NPD or ECD, the sensitivity undoubtedly could be increased further. The repeatability of the retention times for

the analytes was better than 0.16% and the repeatability of peak areas was 4–12%.

4. Conclusions

The RPLC–GC method developed proved to be efficient for the determination of opiates in urine samples. The clean-up was effective and simple; the only manual step in the sample preparation was filtration of the sample. The total time required for analysis was under 60 min, which is much less than with traditional methods. Furthermore, the method was linear and repeatable, and the sensitivity was good.

Acknowledgments

Grants from the Finnish Cultural Foundation and The Finnish Society of Sciences and Letters are acknowledged. The authors also wish to thank the Crime laboratory of the Finnish National Bureau of Investigation for donating the drug standard material.

References

- [1] E.J. Cone, S. Dickerson, B.D. Paul and J.M. Mitchell, *J. Anal. Toxicol.*, 17 (1993) 156–164.
- [2] H. Sachs and W. Arnold, *J. Clin. Chem. Clin. Biochem.*, 27 (1989) 873–877.
- [3] T. Hyötyläinen, H. Sirén and M.-L. Riekkola, *J. Chromatogr. A*, 735 (1996) 439–447.
- [4] P. Wernly and W. Thormann, *Anal. Chem.*, 64 (1992) 2155–2159.
- [5] E. Hufschmid, R. Theurillat, V. Martin and W. Thormann, *J. Chromatogr. B*, 668 (1995) 159–170.

Table 2

Linearities in the range 0.09–15 $\mu\text{g/ml}$, limits of detection (ng/ml) and repeatability of retention times and peak areas (R.S.D., %)

	Dihydrocodeine	Codeine	Ethylmorphine	Morphine	Heroin
Linearity	0.993	0.993	0.900	0.958	0.951
LOD	61	67	90	92	89
Repeatability					
t_r	0.10	0.16	0.15	0.10	0.13
Area	6.5	10.1	4.0	10.9	11.8

Calculated from six replicant runs.

- [6] J. Pothier, N. Galand and C. Viel, *J. Chromatogr.*, 634 (1993) 356–359.
- [7] D. Wielbo, R. Bhat, G. Chari, D. Vidyasagar, I.R. Tebett and A. Gulati, *J. Chromatogr.*, 615 (1993) 164–168.
- [8] R.W. Milne, R.L. Nation, G.D. Reynolds, A.A. Somogyi and J.T. Van Crugten, *J. Chromatogr.*, 565 (1991) 457–464.
- [9] A.S. Christophersen, A. Biseth, B. Skuterud and G. Gadeholt, *J. Chromatogr.*, 422 (1987) 117–124.
- [10] B.H. Chen, E.H. Taylor and A.A. Pappas, *J. Anal. Toxicol.*, 14 (1990) 12–17.
- [11] H.-M. Lee and C.-W. Lee, *J. Anal. Toxicol.*, 15 (1991) 182–187.
- [12] T. Vu-Duc and A. Vernay, *HRC*, 13 (1990) 162–166.
- [13] G.F. Grinstead, *J. Anal. Toxicol.*, 15 (1991) 293–298.
- [14] C.J. Bailey, R.J. Ruane and I.D. Wilson, *J. Chromatogr. Sci.*, 32 (1994) 426–429.
- [15] S. Cardenas, M. Gallego and M. Valcarcel, *Rapid Commun. Mass Spectrom.*, 10 (1996) 631–636.
- [16] U.A.Th. Brinkman, *J. Chromatogr. A*, 665 (1994) 217–231.
- [17] E.C. Goosens, D. de Jong, G. de Jong, F.D. Rinkema and U.A.Th. Brinkman, *J. High Resolut. Chromatogr.*, 18 (1995) 38–44.
- [18] R.E. Majors, *J. Chromatogr. Sci.*, 18 (1980) 571–579.
- [19] M. Saarinen, H. Sirén, and M.-L. Riekkola, *J. Chromatogr. B*, 664 (1995) 341–346.
- [20] T. Hyötyläinen, R. Pilviö and M.-L. Riekkola, *J. High Resolut. Chromatogr.*, 19 (1996) 439–443.
- [21] T. Hyötyläinen, T. Hyvönen and M.-L. Riekkola, *J. Chromatogr. Sci.*, (1997) in press.
- [22] P. van Zoonen, G.R. van der Hoff and E.A. Hogendoorn, *J. High Resolut. Chromatogr.*, 13 (1990) 483–488.
- [23] F. Munari and K. Grob, *J. High Resolut. Chromatogr.*, 11 (1988) 172–176.
- [24] K. Grob, *On-Line Coupled LC–GC*, Heidelberg, Hüthig, 1991, p. 376.
- [25] C. deRuiter, J.H. Wolf, U.A.Th. Brinkman and R.W. Frei, *Anal. Chim. Acta*, 192 (1987) 267–275.